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for Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) The goal of this work is to develop a biomarker profile of prostate cancer risk, based on cell signaling proteins that serve as high-level biomarkers of cellular subsystems; e.g., tissue transglutaminase (TTGase), cadherins or catenins, and gelsolin, G-actin or thymosin-beta. TTGase, cadherins, and G-actin are strong biomarkers of prostate cancer and may be strong biomarkers of field disease and premalignancy. The biomarker profile will identify individuals for prostate cancer prevention and rebiopsy, with high sensitivity and specificity, complementing serum PSA screening that captures 95% of prostate cancer cases with only 20% specificity. Protein biomarkers are quantified in single cells by Quantitative Fluorescence Imaging Analysis (QFIA), which requires stoichiometric labeling of high-quality tissue specimens. A complex and highly integrated configuration of standard operating procedures, tissue culture models, and optimal tissue specimens has been developed and recent implementation supports down regulation of TTGase as a biomarker for prostate premalignancy. Fluorescence signals are currently generated with organic fluorophores but we are now investigating inorganic nanocrystals for biomarker labeling and expect, during the next year of this award, to apply nanocrystal-antibody conjugates to analysis of multiplexed signals from single cells as part of our development of an extensive QFIA data set with prostate specimens.				
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INTRODUCTION

In the U.S., prostate cancer (CaP) affects one in six older men and is the second leading cause of cancer deaths in this population. Though serum PSA is a useful screening tool, only 20% of individuals who meet a threshold concentration that includes 95% of cancer cases, are diagnosed with CaP, consequently, 80% of PSA 'positive' individuals are candidates for biopsy. Further, 20% of individuals with an elevated PSA of 4-10 and a negative biopsy are CaP positive on rebiopsy. The majority of PSA 'positive' individuals who will not develop clinical cancer in their lifetime will be faced with long term repeated biopsy. A minority of PSA 'positive' individuals will experience disease progression until a repeat biopsy is positive. There is an urgent need for effective procedures that risk stratify clinically suspect patients with elevated serum PSA. The purpose of this award is to develop a biomarker profile for fine needle aspirate (FNA) or core cells, which identifies individuals for cancer prevention and rebiopsy and will serve as a guide for development of new therapeutic strategies. High-level biomarkers, including quantitative changes in tissue transglutaminase,¹ G-actin² or gelsolin,³ and catenins or cadherins,⁴ are expressed in histologically normal-appearing areas of cancer-bearing prostates (field effect)⁵ and, in the present award, are being evaluated as indicators of premalignant disease.⁶ Archived tissue blocks from resected glands and archived cores and FNAs from resected glands and patient biopsies are fixed, slide mounted, and stoichiometrically labeled for each of the biomarker proteins and analyzed by single-cell quantitative fluorescence imaging analysis (QFIA).⁷ Acquired data will be analyzed chiefly with ROC curves to determine biomarker cutoffs that will comprise a profile with high sensitivity and specificity for determining prostate cancer risk.

BODY

Work completed to date advances each of the three 'Tasks' delineated in DAMD17-02-1-0121 by providing a) efficient prostate tissue collection procedures, b) optimized tissue processing for Quantitative Fluorescence Imaging Analysis (QFIA), c) standards for identifying archived tissues suitable for QFIA, d) model tissue culture systems for establishing optimum cell fixation and stoichiometric labeling of potential biomarkers of early prostate cancer and premalignant disease, e) standard operating procedures (SOPs) for quality-controlled specimen collection and processing, f) programmed and calibrated instrumentation for quantitative image capturing, g) software instruction sets for high-content quantitative analysis of fluorescence images, h) stoichiometric labeling and quantification of candidate biomarkers of prostate premalignancy, which include nuclear DNA, tissue transglutaminase (tTGase), Gelsolin, beta-Catenin and Thymosin-beta, in tissue sections and single cells, and i) a demonstration of reduced expression of tTGase in epithelial cells of paraffin-embedded, archived prostate specimens.

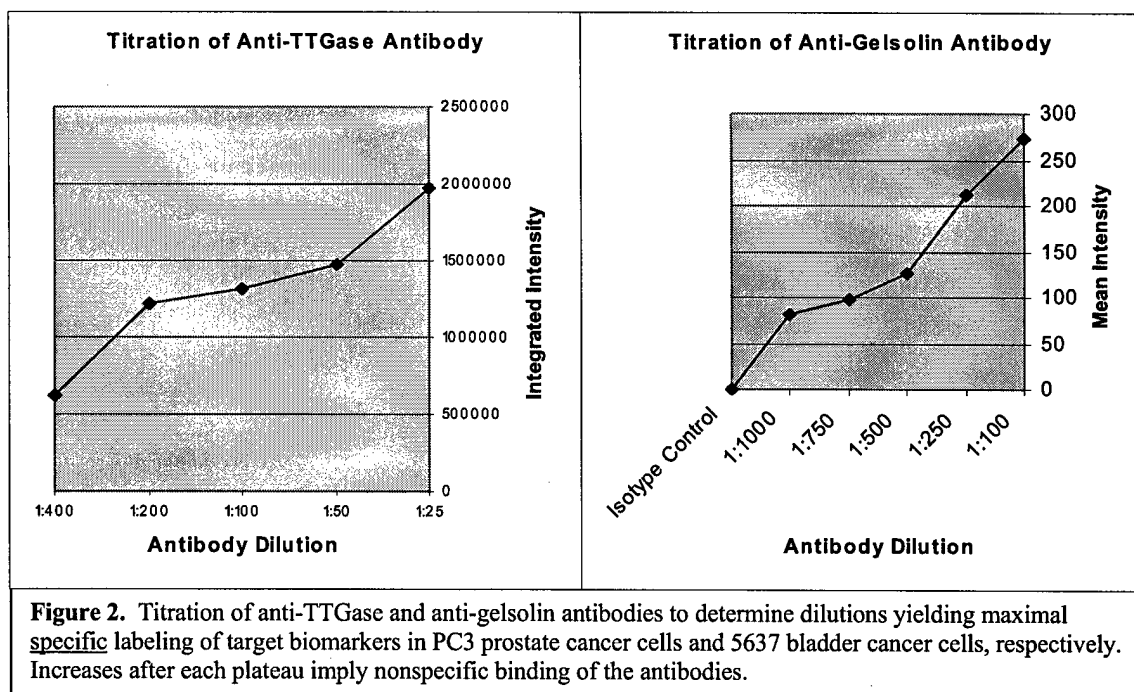
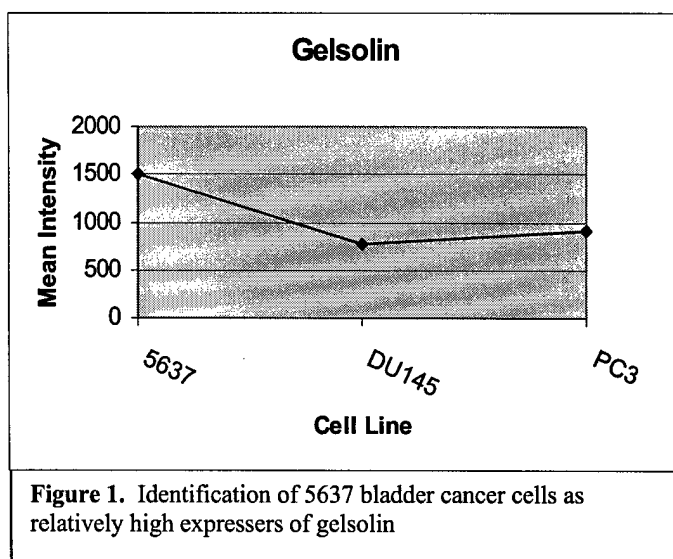
This Progress Report covers a period of eight months, beginning February 7, 2003, the date marking official recognition of DAMD17-02-1-0121 as an active agreement between the Department of Defense and the University of Nebraska Medical Center. Transfer of DAMD17-02-1-0121 from the University of Oklahoma Health Sciences Center to the Nebraska Medical Center necessitated establishing new human studies approvals and documentation. Integrated into this process were 1) an extensive review and refinement of human studies protocols developed for the original submission of this proposal by the University of Oklahoma Health

Sciences Center and 2) negotiations for approval of and development of new documentation for University Hospital, Clarkson Hospital, and the Omaha Veterans Administration Hospital as new sites for acquisition of human tissue specimens for prostate cancer research. Linked to our efforts to secure approvals and develop new documentation for human studies, we established an efficient system of patient consenting, acquisition of high-quality tissues from resected glands, and anonymous registration of processed tissues, for each of the three hospital sites.

QFIA is critically dependent on high-quality tissue specimens that are optimally fixed and have not lost cellular proteins either through 1) autolysis of glands that stand for hours after resection, prior to specimen collection or 2) insufficient fixation of cellular proteins. Recently, we implemented established protocols for specimen collection 15 to 30 minutes after the prostate is separated from the patient, obviating significant autolytic damage to the specimens and physical loss of biomarkers. Fine needle aspirates (FNAs), cores, and tissue blocks are collected systematically according to a Standard Operating Procedure (see **Appendix 1**) developed with the support of award DAMD17-02-1-0121. Additionally, biomarkers may be functionally lost through over fixation and consequent chemical modification of the epitopes of interest or excessive, protein cross-linking that may trap epitopes in a tight matrix relatively impermeable to fluorescence labeling reagents. Loss of biomarker signal caused by over fixation may be compensated by epitope recovery procedures, but not in all cases. Thus, over fixation of specimens collected by our laboratory is avoided. We have established standard, optimal fixation procedures for FNAs and core biopsies, to be stored as suspensions in an ethanol mixture, at -80°C , or in paraffin blocks, respectively. In addition, we have established optimal fixation procedures for tissue blocks to be embedded in O.T.C. for cryosectioning or paraffin for standard sectioning. For previously archived specimens that were not fixed according to our standard procedures, specimens are screened for QFIA by a combination of epithelial eosinophilia relative to stromal eosinophilia and quantitative albumin staining, developed with the present award. The goal is to work with specimens that are not compromised by global loss of proteins including biomarkers of interest. For some cases, core biopsies were found to be superior to sections of corresponding tissue blocks, suggesting efficient fixation of the cores. Compromised tissue blocks may represent areas of a resected gland that were delayed in their exposure to fixative and consequently progressed in the autolytic process with global loss of protein. Based on our experience, we expect that sections of only a small proportion of previously archived tissue blocks may be adequately fixed (e.g., because of proximity to the outer surface of the gland and/or because that particular gland was quickly placed in fixative) and therefore suitable for QFIA. We have identified previously archived cores that exhibit protein loss, but we anticipate that the majority of archived cores will be suitable for QFIA. Screening for archived cores and tissue blocks characterized by the absence of global loss of proteins is integral to our standard procedures for developing biomarkers of early prostate cancer or premalignant disease. Our work responds to studies emphasizing the need for precise specimen processing for each biomarker or combination of biomarkers.

QFIA is based on highly reproducible, stoichiometric labeling of cellular biomarkers with fluorescence reagents. This is accomplished in our laboratory with a programmable, automated instrument (BioGenex *i6000* Autostainer) that individualizes the treatment of each slide in a run. Reagents comprising the fluorescence labeling system are titrated to a plateau of emission for the biomarker under investigation. We have developed programs for automated labeling of cellular

DNA (see **Appendix 2**), tTGase, Gelsolin, beta-Catenin, and Thymosin-beta and have identified cell lines that optimally express each biomarker (**Figure 1**). These cell lines are used for titrating fluorescence labeling systems to determine stoichiometric labeling conditions for the biomarkers of interest (**Figure 2**). The cultured cell lines include PC3, DU-145, and LNCaP prostate cancer cells, and 5637 bladder cancer cells. Cell culture is implemented *via* a quality-controlled tracking system (see **Appendix 3**). Both avidin-biotin-based and fluorophore-coupled-antibody-based labeling protocols have been developed, using these model systems. At present,



our laboratory uses AlexaFluors (organic fluorophores) to generate fluorescence signals, but we are also evaluating inorganic nanocrystals for fluorescence labeling of biomarkers. Nanocrystals exhibit high quantum efficiency and photostability offering the potential for direct coupling with primary antibodies and efficient multiplexed signaling for simultaneous quantitative analysis of multiple biomarkers in single cells. We have implemented a compatible fixation protocol and a fluorescence-labeling program for the new nanocrystal technology, and we are now evaluating the labeling characteristics of streptavidin-conjugated nanocrystals in relation to streptavidin-

conjugated AlexaFluors, with all other labeling conditions held constant. In parallel, we are implementing a scheme we designed for synthesis of antibody-nanocrystal conjugates.

Images of fluorescing cells and tissue sections stoichiometrically labeled for specific protein biomarkers [Figures 3 and 4] are captured with a fully automated fluorescence microscope (Leica DMRXA2) fitted with a digital CCD camera (Hamamatsu ORCA-ER 1394) controlled with ImagePro Plus imaging software (MediaCybernetics). During this period of the DoD Award, we configured the camera-microscope system, and designed computer instruction sets, some packaged as software macros, for both automated and semi-automated capture of fluorescence images. In addition, we completed spatial calibrations of the instrument, and set up quality-control instruction sets to evaluate linearity of the system's response to fluorescent events [Figure 5], linearity of the camera's response to increasing light exposure [Figure 6], and stability of the fluorescence excitation source. We developed instructions sets in the ImagePro Plus environment for segmenting and processing captured images (saved as TIF files) for high-content quantitative analyses of the segmented events, and for transferring the data to Excel files for statistical and graphical analyses.

Our instruction set for monitoring energy output of the Hg vapor lamp detected energy fluctuations in a lamp nearing the end of its useful service (*ca.* 200 hours). Lamp energy fluctuations [Figure 7] can introduce significant variability into our quantitative measurements. As a consequence, we recently

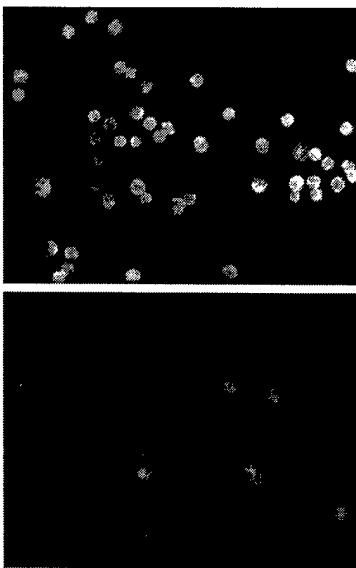


Figure 3. Fluorescence labeling of Gelsolin in 5637 cells (top) and DU-145 cells (bottom)

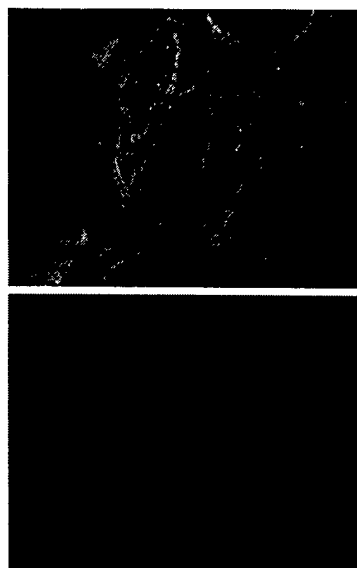


Figure 4. Fluorescence labeling of TTGase in core biopsies of BPH (top) and CaP glands (bottom)

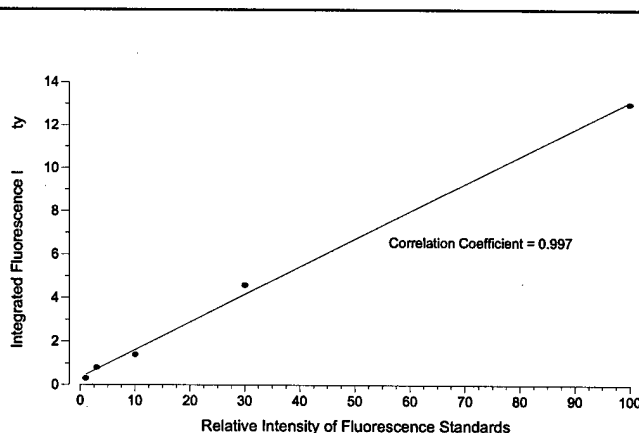


Figure 5. Linear Response of the Leica Imaging Analysis System To Quantitative Fluorescence Standards. Images of standard fluorescent beads (InSpeck Intensity Standards; Molecular Probes) were captured as 'TIF' files, at the same exposure settings. Captured images were segmented and measured with ImagePro Plus software. Data were transferred to Plotit for Windows (Scientific Programming Enterprises) for graphical analysis.

completed a review of equipment for fluorophore excitation in fluorescence microscopy, developed a proposed modification of our Leica system, and entered into an agreement with North Central Instruments to customize our automated microscope with the proposed configuration for fluorescence excitation with an exceptionally stable Hg/Xenon light source (Hamamatsu L7047 with the E7536 lamp housing and C7535 power supply) that has a service life (*ca.* 2000 hours). The equipment will be installed and evaluated in the next three to four weeks. We anticipate a high level of stability required for precise and reproducible measurement of fluorescent events.

A second imaging analysis system was configured and placed into operation, during this past year of our DoD Award. This system, a Laser Scanning Cytometer (CompuCyte Corp.), is based on

laser-excited fluorescence and photomultiplier tube (PMT) mapping of fluorescent events for high-content quantitative measurements with integrated software. As a quality assurance procedure, single-cell samples are analyzed periodically with both the camera-based system and the PMT-based system. Superimposable results obtained with these technologically diverse systems establish the validity of our measurements.

Our work on systems development

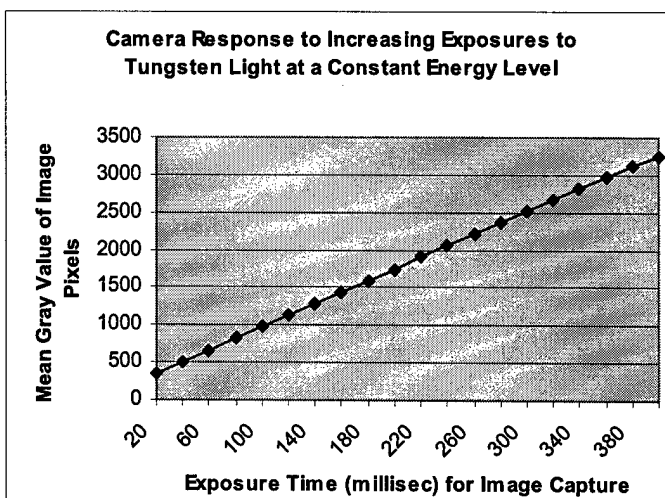


Figure 6. Linear Response of the CCD Camera to Increasing Exposure to Tungsten Light at a Constant Energy Level. Light from the tungsten light was captured as a blank image, at the indicated exposures. An area of interest (AOI) was mapped to the same location of each image and mean value of AOI pixels was determined with ImagePro Plus software. Pixel values were mapped on a 12 bit (0-4095) gray scale.

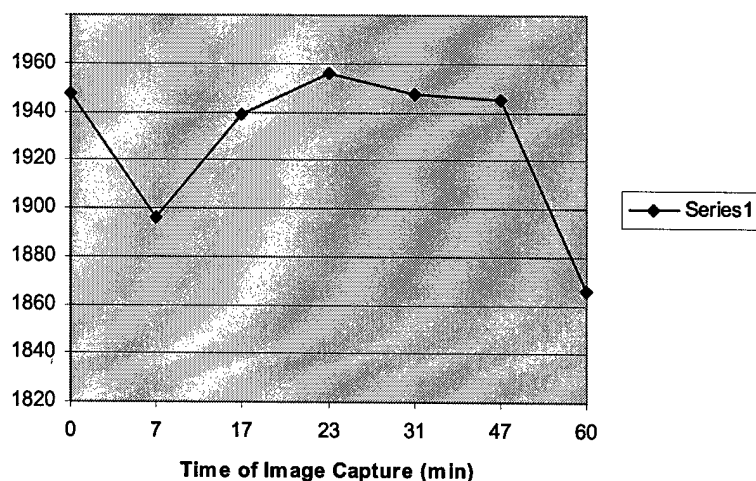


Figure 7. Fluctuations in Energy Output of the Mercury Vapor Lamp of the Leica Automated Fluorescence Microscope. Light from the mercury vapor lamp was captured as a blank image, at the indicated times. An area of interest (AOI) was mapped to the same location of each image and mean value of AOI pixels was determined with ImagePro Plus software. Pixel values were mapped on a 12-bit (0 to 4095) gray scale.

and optimization has culminated in its direct application to systematic evaluation of potential biomarkers of early prostate cancer and premalignant disease in prostate tissue specimens. Our recent evaluation of tTGase expression in cancerous and noncancerous prostate glands confirmed down regulation of this protein biomarker in prostate cancer (see **Figure 4 and Appendix 4**). Age-matched archived blocks from six prostate cancer (CaP) cases and six benign prostatic hyperplasia (BPH) cases were sectioned, slide mounted, deparaffinized, processed for epitope retrieval, and stoichiometrically labeled for tTGase with an optimized avidin-biotin system. Fluorescent events were captured with the Leica automated microscope system and analyzed with ImagePro Plus software. Descriptive statistics and probability measurements were completed in the Excel environment (Microsoft). The frequency of labeled glands in the BPH specimens (41%) was significantly ($P < 0.05$) higher than the frequency in cancer-bearing glands (17%). Of particular interest is our finding that the mean fluorescence intensity of positive glands in BPH specimens was significantly ($P < 0.001$) greater than mean fluorescence intensity of glands in either the normal-appearing areas or cancerous areas of cancer-bearing specimens. These findings support the concept of field effect or field disease, and the application of QFIA to single-cell proteomics for establishing biomarker profiles that identify patients with early prostate cancer or premalignant disease.

KEY RESEARCH ACCOMPLISHMENTS

- Established new human studies approvals and documentation as part of the transfer of Award Number DAMD17-02-1-0121 from the University of Oklahoma Health Sciences Center to the University of Nebraska Medical Center.
- Reviewed and refined human studies protocols associated with DAMD17-02-1-0121, to meet evolving national standards and newly implemented HIPPA requirements for protection of human subjects involved in medical research.
- Negotiated approvals and established documentation for University Hospital, Clarkson Hospital, and the Omaha Veterans Administration Hospital as sites for acquisition of human tissue specimens for prostate cancer research.
- Established a system of patient consenting and tissue acquisition and archiving for the three sites currently integrated into our human studies protocol.
- Established a Standard Operating Procedure for systematic collection of FNAs, cores, and tissue blocks from resected prostate glands (see Appendix 1).
- Determined optimal fixation procedures for preparing cores and tissue blocks for O.T.C. and paraffin embedding preceding sectioning and slide preparation for Quantitative Fluorescence Imaging Analysis (QFIA).
- Developed a protocol for dissociation of FNA fragments to single cells for fixation as cell suspensions, preceding imprinting to slides for QFIA.

- Collected, processed and archived prostate specimens including FNAs, core biopsies, and tissue blocks from resected glands of 21 patients.
- Implemented a quality-controlled tissue culture program (see Appendix 2) for cell lines that serve as models to establish stoichiometric fluorescence labeling for QFIA of biomarkers in single cells.
- Developed avidin-biotin-based and fluorophore-coupled-antibody-based fluorescence labeling protocols for quantification of tissue transglutaminase (tTGase), Gelsolin (replaces G-actin), and beta-Catenin (replaces pan cadherins) in prostate tissue sections and single-cell suspensions.
- Programmed and calibrated a photomultiplier-based Laser Scanning Cytometer and CCD camera-based Automated Fluorescence Microscope for image capture as a first step in QFIA of the marker proteins tTGase, Gelsolin, and beta-Catenin in prostate tissue samples.
- Using ImagePro Plus software, developed programs for high-content quantitative analysis of images captured by the image capture programs of the Automated Fluorescence Microscope.
- Demonstrated a statistically significant down regulation of tTGase in sub optimum, archived cores from cancerous and non-cancerous prostate glands.
- Established a standard for identification of tissue specimens optimum for QFIA.
- Work on implementing a nanocrystal technology for rapid and efficient multiplexing of fluorescence emissions for simultaneous QFIA of three or more single-cell biomarkers.

REPORTABLE OUTCOMES

Abstracts

1. George P. Casale, Chantey Morris, George P. Hemstreet, III. A Monoclonal Antibody for Quantitative Single-Cell Analysis of Protein Adducts of Estradiol-3,4-quinone, a Suspected Initiator of Non-Familial Prostate Cancer. 82nd Annual Meeting of the South Central Section (SCS) of the AUA, Inc., September 6-10, 2003. Boston, Massachusetts.
2. George P. Hemstreet, III. Genotypic and Phenotypic Biomarker Profiles for Individual Risk Assessment. 82nd Annual Meeting of the South Central Section (SCS) of the AUA, Inc., September 6-10, 2003. Boston, Massachusetts.
3. Dali Huang, George P. Casale, Nizar K. Wehbi, George P. Hemstreet, III. Tissue Transglutaminase Down-regulation: a Potential Biomarker for Prostate Cancer Premalignancy. American Association for Cancer Research (AACR), January 25-29, 2004, Waikoloa, Hawaii. (Submitted)

4. George P. Casale, Nizar K. Wehbi, Dali Huang, Chantey Morris, George P. Hemstreet, III. A Monoclonal Antibody for Macromolecular Adducts of Estradiol-3,4-quinone, a Suspected Initiator of Breast and Prostate Cancers. American Association for Cancer Research (AACR), March 27-31, 2004, Orlando, Florida. (Submitted)
5. George P. Hemstreet, III, George P. Casale, Nizar K. Wehbi, Dali Huang. Phenotypic Biomarker Profiles for Individual Risk Assessment. American Urological Association (AUA), May 8-13, 2004, San Francisco, California. (Submitted)

CONCLUSIONS

A specifically targeted configuration of standard operating procedures, tissue culture models, and optimal tissue specimens has been established for quantitative analysis of protein biomarkers of prostate cancer and premalignant disease, in single cells of the prostate. Work completed to date has advanced each of the three 'Tasks' delineated in DAMD17-02-1-0121 by providing a) efficient prostate tissue collection procedures, b) optimized tissue processing for Quantitative Fluorescence Imaging Analysis (QFIA), c) standards for identifying archived tissues suitable for QFIA, d) model tissue culture systems for establishing optimum cell fixation and stoichiometric labeling of potential biomarkers of early prostate cancer and premalignant disease, e) standard operating procedures (SOPs) for quality-controlled specimen collection and processing, f) programmed and calibrated instrumentation for quantitative image capturing, g) software instruction sets for high-content quantitative analysis of fluorescence images, h) stoichiometric labeling and quantification of candidate biomarkers of prostate premalignancy, which include nuclear DNA, tissue transglutaminase (tTGase), Gelsolin, beta-Catenin and Thymosin-beta, in tissue sections and single cells, and i) a demonstration of reduced expression of tTGase in epithelial cells of paraffin-embedded, archived prostate specimens.

We anticipate, during the next year of this award, multiplexing fluorescence emissions from multiple biomarkers stoichiometrically labeled with nanocrystal-primary antibody conjugates, allowing simultaneous measurement of these biomarkers in single cells. The next year of this award also will be characterized by extensive application of our newly developed infrastructure to quantitative analyses of biomarkers in tissue sections, cores, and FNAs of archived specimens collected from cancer patients and BPH patients and other non-cancer controls. The accumulated information will permit a direct analysis of the sensitivity and specificity of each biomarker for detecting prostate cancer and premalignant disease, an objective one step removed from our ultimate goal of developing a biomarker profile that will identify individuals for cancer prevention and rebiopsy and serve as a guide for development of new therapeutic strategies. Software (MediaCybernetics) and hardware (Leica Automated Microscope and CompuCyte Laser Scanning Cytometer) are commercially available for widespread application of these emerging biomarker profiles.

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Hoechst Staining of PC3 Cell Line

Prepared by: Nizar Wehbi
Date: February 10, 2003
Updated: August 20, 2003

Materials and Vendors

Reagent	Vendor	Cat #
MOPSO	Sigma-Aldrich	<u>M8389</u>
Potassium chloride	Sigma-Aldrich	<u>P3911</u>
EDTA Dipotassium ethylenediamine tetra-acetic acid	Sigma-Aldrich	<u>ED2P</u>
Sodium azide	Sigma-Aldrich	<u>S8032</u>
Potassium hydroxide	Sigma-Aldrich	<u>P5958</u>
0.5 um Whatman Nucleopore 25mm	Fisher scientific	<u>0930065</u>
95% non-denatured Ethanol	General Supply	<u>103479</u>
Sodium Chloride	General Supply	<u>103507</u>
Hoechst 33258	Molecular Probes	<u>H-21491</u>
10% methanol-free EM grade formaldehyde	Polysciences, INC	<u>04018-4</u>
Isoton II diluent	Fisher scientific	<u>23-375212</u>
Accuvette II.	Fisher scientific	<u>NC9720669</u>
Carboxyfix-E	StatLab	<u>CF 100-4</u>
Polyethylene Glycol	Sigma	<u>P-5402</u>
Disodium EDTA	Sigma	<u>ED2SS</u>
Phosphate Buffered Saline (PBS)	GIBCO	<u>14040-141</u>
0.22 Magna Nylon Filter 47 mm	Fisher	<u>R02SP04700</u>
NPG Propyl Gallate	Sigma	<u>P3130-100G</u>
Glycerol, 99.5%, Spectrophotometric	Sigma	<u>191612-1L</u>
Trizma® Pre-set crystal, pH 8.0	Sigma	<u>T8443</u>

Preparation of Solutions and Reagents

Preparation of QFIA Fixit:

Preparation of 10X MOPSO Buffer:

450.8 g 3-[morpholino]-2 hydroxypropanesulfonic acid (MOPSO) (Sigma-Aldrich) and 298.2 g KCl dissolved in 3630 mL double-distilled H₂O. Dissolve thoroughly and freeze in precisely 400 mL volumes.

Preparation of Working Buffer (for 8 L of QFIA Fixit):

Thaw 400 mL 10X MOPSO and combine with 3600 mL deionized double-distilled H₂O, 14.74 g dipotassium ethylenediamine tetra-acetic acid (EDTA) and 0.8 g sodium azide. Dissolve thoroughly and adjust pH to 6.5 with 10M KOH.

Preparation of QFIA Fixit (for 4 L of QFIA Fixit):

Filter 1896 mL working buffer through a 0.22 micron Magna Nylon Filter (Fisher Scientific cat # R02SP047000). Filter 2104 mL 95% non-denatured EtOH (UNMC General Supply) through another Magna nylon filter.

Combine the filtered solutions and mix thoroughly. Store for up to one year at either room temperature or at 4° C.

Preparation of cell adherent fluid

Dissolve 7.36 g dipotassium EDTA and 0.8 g sodium azide in 4000 mL double-distilled H₂O

1. Adjust the pH to 5.5 with KOH
2. Filter and store at -20° C

When needed, thaw and adjust pH

Preparation of Modified Saccomanno Fixative (Immunofix)

- **Buffer 'A'** (0.1 M citric acid): 5.25 g citric acid dissolved in 250 mL double-distilled H₂O. Refrigerate
- **Buffer 'B'** (0.2 M dibasic sodium phosphate): 7.1 g anhydrous sodium phosphate (dibasic) and 250 mL double-distilled H₂O. Refrigerate
- **Buffered-filtered Saline (BFS, 1 L)**
 1. 9.0 g NaCl, 980 mL double-distilled H₂O, and 20 mL Buffer 'B' (warmed until crystals have dissolved)
 2. Adjust the pH to 7.0 with Buffer 'A'

3. Filter and refrigerate. Discard after one week

Modified Saccomanno Fixative (Immunofix)

1. 20 mL Polyethylene glycol (PEG) 1450 (**Sigma-Aldrich**), 516 mL 95% EtOH and 464 mL BFS
2. Melt PEG at 60° C
3. Introduce a stirring bar into the EtOH/BFS mixture, begin stirring
4. Slowly add 20 mL of melted PEG to the stirring solution, then let stir for 1 hour
5. Store at room temperature

Preparation of Hoechst 33258 working solution (10 uM alcoholic Hoechst, 40.0 mL)

1. 10X MOPSO/NaCl:

1. Dissolve 233.76 g NaCl and 45.08 g MOPSO in 4000 mL double-distilled H₂O
2. Store precisely 400 mL volumes at -20° C

2. MOPSO/EDTA:

1. Thaw 400 mL 10X MOPSO/NaCl and combine with 3600 mL double-distilled H₂O
2. Dissolve 14.88 g sodium EDTA
3. Adjust the pH to 6.8, filter
4. Store approximately 500 mL volumes at -20° C

3. Hoechst working solution: combine

1. 0.2 mL Hoechst stock solution (2.0 mM)
2. 29.3 mL MOPSO/EDTA (pH 6.8)
3. 10.5 mL 95% Ethanol

Preparation of Hoechst Stock Dye Solution

1. Hoechst 33258 is prepared as stock 2.00 mM solutions.
2. Using a microbalance capable of measurement to at least ± 0.1 mg, arbitrarily weigh out 100-200 mg H-33258 to at least the nearest 0.1 mg. This means to "dump" a spatula of dye powder onto a weighing paper on the balance and weigh it accurately.
The measured weight of Hoechst is used to calculate the volume of double-distilled water to be added according to the following:

$$\text{Vol. Of Water (ml)} = \left(\frac{\text{Wt. of Hoechst (mg)}}{\text{Mol. Wt of Hoechst (mg/mmol)}} \right) / \left(\frac{2 \text{ mmol}}{1000 \text{ ml}} \right)$$

Molecular Weight of Hoechst= 623.96 mg/mmol.

In other words, **Vol. Of water (ml)= Wt of Hoechst (mg) x 0.801333 (ml/mg)**

3. Place the water in a Teflon bottle then carefully pour the Hoechst dye into the bottle and allow it all to dissolve. Bottles should be washed with double-distilled filtered water. A bottle should only be used for a single dye, that is do NOT place another dye in a bottle that previously held Hoechst 33258.
4. Check the concentration of the stock dye solution by spectrophotometry. To do this, transfer 50.0 μ L of dye to a disposable clear plastic centrifuge tube and add 9.94 mL of 95 % ethanol and 10 μ L of 6 N HCl. Mix thoroughly and read the absorbance in a spectrophotometer. The wavelength is 345 nm for H-33258. The absorbance for H-33258 should be 0.366 ± 0.025 (0.341 - 0.391). If the dye falls outside of these limits, check that the dye was all dissolved, that the calculations were correct, that the dilution in ethanol was correct and that the dye is not wet or changed color. If no source of error is apparent, start over.
5. Dyes should be kept under refrigeration in foil-wrapped Teflon bottles and should only be entered with a fresh, clean pipette for the purpose of preparing a working solution. Contamination of the stock requires that it be discarded. It should NEVER be left on the benchtop with the top removed. The concentration can be checked spectrophotometrically as described above.
6. Label the bottle clearly with: the dye, its concentration, the date prepared, the initials of the person preparing the dye, and the absorbance measured.
7. Record absorbance measured in the Excel Spreadsheet and determine that the readings are within standard deviation of previous results.

NPG MOUNTING MEDIA

Combine:

1.91g NPG (n-propyl Gallate Sigma #P-3130)

90ml Glycerol (spectranalyzed grade) (112.5g)

Let stir overnight on speed 3 cover beaker with foil
then add 10mls of Trizma buffer slowly while stirring.

TRIZMA BUFFER:

0.709g preset Trizma Crystal (pH 8.0)

100mls DDW

Stir until dissolved

Filter into an autoclaved 100ml bottle

Once Trizma buffer has been added to Glycerin then let stir another 15 minutes more. Then transfer into an autoclaved 100ml bottle that has been wrapped in aluminum foil (to protect from light). May be used 24 hours later.

Store up to one month at room temp.

PROTOCOL

Fixing PC3 Cultured Cells For QFIA

Refer to Tissue Culture Protocol for getting PC3 cells
1 through 7 are done under aseptic techniques

1. Centrifuge cells at 600g for 10 minutes in their media. (Do not forget to balance the tubes)
2. Discard the supernatant
3. Add 1-2 ml of PBS
4. Break up lumps with a 9" glass Pasteur pipette
5. Raise volume to 5 ml
6. Repeat 3 times
7. Count with Coulter Counter
 - a. Put 20 ml isoton in Coulter Cuvet
 - b. Add **100 ul** of cell suspension
 - c. Set the counter to count above the 10um limit
 - d. Take 3 counts and get the average
 - e. This count is in 0.5 ml
 - f. Calculate the total number of cells.

$$\text{Total cells/ml of sample} = \text{Av. Cells counted} \times 400$$

Refer to the attached excel table for calculation

	Count 1	Count 2	Count 3	
	1,224	1,271	1,202	
Av. Cell Count				1,232
Total Cells/ml	(Av. Cell Count x 400)			492,933

8. Add 0.25 ml of 10% EM grade methanol free formaldehyde (Polysciences, Inc.). We want a 0.25 % Formaldehyde after step 15
9. Put in the tube inverter and set it to 20 percent at room temp for 15 mins.
10. Add QFIA FIXIT in a volume equal to the volume of the sample (5 ml)
11. Allow to fix overnight at 4°C prior to freezing or slide preparation.

Slide Preparation

The goal of slide preparation for QFIA is to prepare a non-overlapping monolayer of cells with good cytomorphology and biomarker preservation with minimal loss of cells during processing. In contrast to many applications, air-drying of slides must be avoided because it induces artifacts.

SLIDE IMPRINTING WITH CELLS

1. Thaw a 5-mL sample stored at -80°C and allow the sample to warm to room temperature
2. Place a 25 mm polycarbonate filter (Nucleopore, **Fisher Scientific**) (5.0 μm) onto the scintered glass bed of the filtration unit (**Fisher Scientific**) and clamp the filtration funnel in place
3. Place the unit into a side-arm flask connected to a vacuum line
4. Mix the sample and transfer the sample to the filtration funnel
5. Gently vacuum sample (use the minimal vacuum to get a continuous flow) until 2 mm solution remains (break vacuum)
6. Wash the cells with *ca.* 15 mL of cell adherent solution (leave 2mm solution)
7. Add *ca.* 5 mL of modified Saccomanno fixative to the funnel and mix gently
8. Let the cells stand for 2 min in the fixative
9. Label 1 OptiPlus (3 x 1/3) slides (**BioGenex**)
10. Resume filtering, do not let the filter dry out
11. Lift filter off the scintered glass with a forceps and place on the labeled glass slide, with cell side facing down
12. Gently press down with a moistened Kimwipe, for 7 sec
13. Lift filter off the slide and spray the slide 1 time with Carbofix-E (**StatLab Medical Products**)
14. Let the slide dry for at least 15 min before storing at -20°C
15. Slides may be stored for a maximum of 2 weeks, at -20°C

Hoechst Staining

AutoStainer Setup

BioGenex i6000™ Automated Staining System

1. Turn on the Biogenex i6000
2. Choose "Open Format"
3. Insert "Hoechst Protocol" to the slides to be stained
4. Insert "Blank Slides" for background subtraction
5. Refer to "Hoechst Working Solution QC" Excel sheet for preparation of Hoechst and the Blank Working Solution
6. Start the run

Hoechst Staining Protocol

1. Optimax buffer 2 min; room temp
2. Optimax wash
3. Optimax buffer 2 min; room temp
4. Optimax wash
5. Optimax buffer 2 min; room temp
6. Optimax wash
7. Hoechst stain 1 min; room temp
8. Hoechst stain 1 min; room temp
9. Hoechst stain 1 min; room temp
10. Hoechst stain 1 min; room temp
11. Hoechst stain 1 min; room temp
12. Hoechst stain 1 min; room temp
13. Optimax wash
14. Mount w/ NPG Mounting Media
 - a. Put a drop of NPG Mount on the far side of the slide
 - b. Use a fine forceps for holding the cover slip
 - c. Gently and slowly let far ends of the slide and the cover slip touch
 - d. Lower slowly the other end of the cover slip until they both adhere
15. Let the slides excess solution dry
16. NO NEED FOR SEAL

Notes and Comments

(Please indicate date and writer)

- Do not use a lot of cells in the PBS. Formaldehyde will cross link the cells together and you will end up with clumps in your suspension.
- First Experiment 02/19/2003. Av. Cell Count = 1,232. Total/ml = 489,600/ml ie around 2.5 million in the sample. After adding the Formaldehyde, clumping of cell was observed after 10 min of Formaldehyde application.

-

Quality Control



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PROSTATE TISSUE COLLECTION AND PROCESSING: FINE NEEDLE ASPIRATES (FNA), CORE BIOPSIES, AND TISSUE SECTIONS

Prepared by: Dali Huang
Date of Preparation: June 10, 2003



Materials and Vendors

<u>Substance</u>	<u>Vendor</u>	<u>Cat#</u>
Biopty gun		
Biopty needles (18 GA)	WMT	<u>ACN1820UC</u>
MOPSO	Sigma-Aldrich	<u>M8389</u>
10% methanol-free EM grade formaldehyde	Polysciences, INC	<u>04018-4</u>
Potassium chloride	Sigma-Aldrich	<u>P3911</u>
Dipotassium ethylenediamine tetra-acetic acid	Sigma-Aldrich	<u>ED2P</u>
Sodium azide	Sigma-Aldrich	<u>S8032</u>
Potassium hydroxide	Sigma-Aldrich	<u>P5958</u>
Millipore nylon filter	Fisher scientific	<u>GNWP04700</u>
95% non-denatured Ethanol	General Supply	<u>103479</u>
Sodium Chloride	General Supply	<u>103507</u>
Syringe (20cc)	General Supply	<u>113406</u>
Needles 22 guage 1-1/2 "	General Supply	<u>113390</u>
Cryogenic vial	Fisher scientific	<u>5000-0020</u>
6 ml Falcon tube	General Supply	<u>103902</u>
Scalpel	General Supply	<u>104119</u>
Isoton II diluent	Fisher scientific	<u>23-375212</u>
Accuvette II.	Fisher scientific	<u>NC9720669</u>
Freezer cardboard box	ThermoForma	<u>820002</u>



Preparation of Reagents and Solution

Preparation Of QFIA Fixit:

Preparation of 10XMOPSO Buffer:

450.8 g 3-[morpholino]-2 hydroxypropanesulfonic acid (MOPSO) and 298.2 g KCl dissolved in 3630 mL double-distilled H₂O. Dissolve thoroughly and freeze in precisely 400 mL volumes.

Preparation of Working Buffer(for 8 L of QFIA Fixit):

Thaw 400 mL 10X MOPSO and combine with 3600 mL deionized double-distilled H₂O, 14.74 g dipotassium ethylenediamine tetra-acetic acid (EDTA) and 0.8 g sodium azide. Dissolve thoroughly and adjust pH to 6.5 with 10M KOH.

Preparation of QFIA Fixit(for 4 L of QFIA Fixit):

Filter 1896 mL working buffer through a 0.20 micron white nylon filter. Filter 2104 ml of 95% non-denatured Ethanol through another 0.2 micron white nylon filter. Combine the filtered solutions and mix thoroughly. Store for up to one year at either room temperature or at 4° C.

10 X MOPSO (for QFIA Fixit)										Main	
Component	Vendor	Cat. #	Lot #	Expiration Date	Date Opened	Quantity	Units	Reagent Prep. Date	Reagent Exp. Date		
Double-distilled H ₂ O						1815	mL				
3-[morpholino]-2 hydroxypropanesulfonic acid (MOPSO)	Sigma-Aldrich	M-8389		NI		225.4	g	Prepared by:			
KCl	Sigma-Aldrich	P-3911		NI		149.1	g	Date:			
Comments:	Labeling: • Store at -20 C • Store in 400 mL units • Do not re-freeze thawed reagent. • Prepared by:										

QFIA Fixit (for 4 L)										Main	
Component	Vendor	Cat. #	Lot #	Expiration Date	Date Opened	Quantity	Units	Reagent Prep Date	Reagent Exp Date		
Working buffer (PH 6.5)	10XMOPSO					200	mL				
	Double-distilled H ₂ O					1800	mL				
	Dipotassium ethylenediamine tetraacetic acid(EDTA)	Sigma-Aldrich	ED2P			7.37	g	Prepared by:			
	Sodium azide	Sigma-Aldrich	S8032			0.4	g				
	Potassium hydroxide	Sigma-Aldrich	P5958			4~5mL 10M KOH adjust to PH6.5	Date:				
Filter 1896 mL working buffer through a 0.22um Magna nylon filter											
95% non-denatured Ethanol	UNMC general Supply	103479				2104	mL				
Filter ethanol through a 0.22um Magna nylon filter and combine with 1896 mL filtered buffer from above, mix thoroughly.											
Comments: • Store at room temperature or 4°C • Can be stored for up to one year	Labeling: • Reagent • Prep date • Exp date • Prepared by:										



Tissue Acquisition Procedures

- Patient should consent against the IRB protocol #131-02-EP by read and sign the consent form before his scheduled surgery.
- The original informed consent form is kept in a secure location, the office of Registration Coordinator, Diana Axiotis, P.A..
- One copy of patient consent form is submitted to Patient's Record.
- Another copy of patient consent form is faxed to the UNMC Tissue Procurement Facility Committee (Pamela Althof, Fax: 402-559-7248 or 402-559-6018, Pager Number for Pam: 888-0360) one day prior to the scheduled surgery with the completed tissue request form and estimated tissue amount to be obtained.



Tissue Procurement Request Form

Prior to release and distribution of specimens, investigators must submit the following request form for approval to the Tissue Procurement Core Facility Committee:

Request Form for Tissue Procurement

Investigator: George P. Hemstreet, III, MD, Ph.D

IRB Approval # or Exempt Status # (This must be provided) 131-02-EP

Funding Source and Principal Investigator

Department Of Defense Grant (DOD)

PI: George P. Hemstreet, III, MD, Ph.D

Specimen Requirements:

Starting and ending dates of study Dec.31, 2002 – Dec.31, 2005

Total # of specimens desired 500 prostate specimen

Specimen/Tissue type(s) FNA, Core biopsy and tissue pieces of
prostate gland

Anatomical site(s) prostate gland

Minimum size/quantity of specimen (i.e. lug, 1cm³) FNA
cells/14

Circle ALL acceptable:cx

- | | | |
|------------------|-------------|----------------|
| 1 . Fresh tissue | Snap frozen | Formalin fixed |
| 2. Primary | Recurrent | Metastatic |

Patient Demographics:

1. Sex male
2. Age (range) 25-100



Additional Procedures Requested:

(Examples include frozen sections, H&E or unstained tissue sections, immunohistochemistry or electron microscopy, cell culture, and cytogenetic analysis.)

Please describe:

None.

Participant Information: (Name, Phone #, Pager # of whom to contact)

Name: Dali Huang

Phone: 9-8255

Pager:

Sample

How to store specimen if immediate delivery is not possible:

N/A

***Note :** The following grant numbers should be acknowledged in publications:

NCI Cancer Center Support Grant P30 CA36727

Nebraska Department of Health Institutional LB595 Grant for
Cancer and Smoking Disease Research

Please submit all applications to:

Pamela Althof, B.A. (Laboratory Supervisor)
c/o Julia A. Bridge, M.D.
University of Nebraska Medical Center
985454 Nebraska Medical Center
Omaha, NE 68198-5454
Phone: 402-559-5733(lab); 402-559-8135 (office)
Fax: 402-559-7248 or 402-559-6018



From: Diana Axiotis, P.A.

To: Pamela Althof, B.A.

Urology Surgery

Coordinator of Tissue

Bank

Extension: 9-4683

Extension: 9-5733

Fax: 9-6529

Fax: 9-7248

Pager: 888-2595

Pager: 888-0360

DATE: _____

Patient Name: _____

IRB Approval: 131-02-EP

Funding Source and Principal Investigator:
Department Of Defense (DOD) Grant

PI: George P. Hemstreet, III, MD, Ph.D

ESTIMATED TISSUE AMOUNT:

- Six core biopsy from prostate gland
- Six Fine Needle Aspirate (FNA) from prostate gland
- Seven pieces of prostate gland tissue, each weigh 200-300 mg

Total amount of prostate gland will weigh about 2 gram.



PROTOCOL

Material Preparation

- Biopsy gun
- 10 Biopsy needles (18 GA)
- 6 of 6ml Falcon tube containing 4 ml of 10% Formaldehyde pre-labeled with case code and
 - (1) CT1 (core tissue, right apex)
 - (2) CT2 (core tissue, right midgland)
 - (3) CT3 (core tissue, right bass)
 - (4) CT4 (core tissue, left bass)
 - (5) CT5 (core tissue, left midgland)
 - (6) CT6 (core tissue, left apex)
- 6 of 1.5ml Eppendorf tubes containing 1ml of digestion buffer with EDTA pre-labeled with case code and
 - (1) FNA1, (right apex)
 - (2) FNA2, (right midgland)
 - (3) FNA3, (right bass)
 - (4) FNA4, (left bass)
 - (5) FNA5, (left midgland)
 - (6) FNA6, (left apex)
- 6 of 50ml Cryogenic vials pre-labeled with case code and
 - 1) T1, (right apex)
 - 2) T2, (right midgland)
 - 3) T3, (right bass)
 - 4) T4, (left bass)
 - 5) T5, (left midgland)
 - 6) T6, (left apex)
- One tube containing 40 ml of 10% methanol-free EM grade formaldehyde
- One tube of 40 ml of 1XMOPSO and one tube of QFIA Fixit
- 10 Syringe (20cc)
- 10 Needles 22 guage 1-1/2 "
- Some 6ml Falcon tubes and 1.5 Eppendorf tubes and 50 ml vials unlabeled for back up
- One box of ice
- OCT and some dry ice
- One marker
- Clinical information forms, including report of the transrectal ultrasonogram
- Surgical pathology/cytology reports from previous biopsies/as



Procedure for CORE BIOPSY

1. Identify the location/zone from which core biopsies will be taken.

- (1) CT1 (core tissue, right apex)
- (2) CT2 (core tissue, right midgland)
- (3) CT3 (core tissue, right bass)
- (4) CT4 (core tissue, left bass)
- (5) CT5 (core tissue, left midgland)
- (6) CT6 (core tissue, left apex)

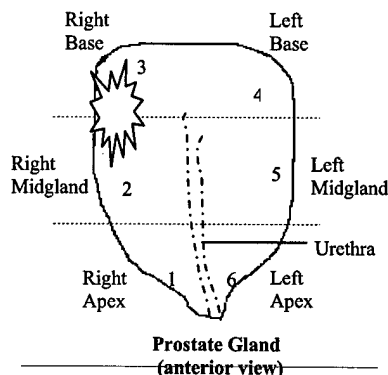
2. Locate needle to the tissue (tumor nodule if palpable or suspicious area identified by transrectal ultrasound) and press the gun.

3. Eject the core biopsy into the 4 ml of 10% Formaldehyde collection vial prelabeled with case code and tissue code.

4. Discard needles. Replace the needle after each location/zone is sampled.

5. Complete the appropriate QFIA and Pathology forms. Record the Pathology Accession number on the QFIA form.

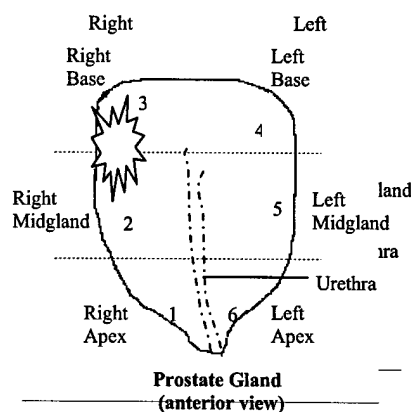
6. The core biopsies are then sent to the Department of Pathology for histological evaluation.





Procedure for FINE NEEDLE ASPIRATES (FNA)

1. Assemble 10cc syringe with 18-gauge 1-1/2 needle.
2. Aspirate a few cc of 1xMOPSO and eject fluid to wet the inner surface of syringe.
3. Aspirate air (about 2cc) into syringe.
4. Insert needle into one of the following tissue target site and draw back on the shaft to about 8cc creating a strong negative pressure.
 - 1) Tumor
 - 2) Area adjacent to the tumor
 - 3) Normal/hyperplastic prostate from an ipsilateral periurethral region
 - 4) Tumor (if present) from contralateral region
 - 5) Normal/hyperplastic prostate from a contralateral region
 - 6) Sextant FNA and core biopsies from the peripheral zone of the right and left apex, midgland and base
5. Direct needle in at least 5 different tissue planes to optimize sampling while maintaining negative pressure. Do not aspirate air.
6. Empty syringe contents into vial containing 0.95 ml of 1 x MOPSO. Discard needle and syringe. Aspirate about 0.6 ml of the 1x MOPSO into the syringe and blow out rapidly several times to rinse most of the cells out of the needle track and barrel. Shake well, and then add 0.05 ml of 10% methanol-free EM grade formaldehyde.
7. Complete FNA from all the other sites.
8. Incubate the specimen with 0.5% formaldehyde at room temperature for 15 min, and then add an equal volume of QFIA fixative to the specimen vial.
9. Complete QFIA form, record the Pathology Accession number and refrigerate samples immediately.
10. FNA cells are sent to the QFIA laboratory (402) 559-4623 and fixed **overnight** at 4°C.





Counting and cryopreservation of core biopsy cells and FNA cells

1. After **overnight** fixation, allow samples to warm to room temperature.
2. Count the cells with a Coulter model Z₂ particle counter. (see next page Coulter counting form)
 - Parameter setting for the counting includes >5um, >10um, >15um.
 - Count twice for each background and cell sample with each parameter.
 - Count the background before each cell sample count. Pipet 20 mL Isoton into an Accuvette II. Do the background count first.
 - Count the sample. Pipet 100 uL of cell sample from 2 ml well-mixed specimen into the 20 mL of Isoton solution, mix thoroughly and count the cells.
 - Wash Coulter probe with Isoton after each cell sample count.
 - Calculate the cell number each vial contains.
Cell number = cell count x 40 x 20 = cell count x 800
3. Count the cells with Hemacytometer and microscope.
 - Pipett 20 ul from 2 ml of well-mixed specimen into Hemacytometer, wait until cells settle down.
 - Count number of high quality single cells.
 - Count number of damaged single cells.
 - Count cells in aggregate.
 - Calculate cell number that each specimen contains.
Cell number = cell count x 10⁴ x 2
4. Evaluate cell count from both sources.
5. Divide the specimen with each pre-labeled Cryogenic vial contains 90,000 cells.
6. Proceed with the QFIA protocol or stored the samples at -80°C.



Coulter counting form:

TissueCode/ Background	>5um		>10um		>15um	
	1st Count	2nd Count	1st Count	2nd Count	1st Count	2nd Count
Background CC1						
Background CC2						
Background CC3						
Background CC4						
Background CC5						
Background CC6						
Background FNA1						
Background FNA2						
Background FNA3						
Background FNA4						
Background FNA5						
Background FNA6						



Tissue Vial Labeling and Storage

1. Each vial of tissue is labeled with

- case number
- tissue code
- storage date

<u>Tissue Description</u>	<u>Tissue Code</u>
FNA, Tumor	FNA1
FNA, Area adjacent to the tumor	FNA2
FNA, Normal/hyperplastic prostate from an ipsilateral periurethreal region	FNA3
FNA, Tumor (if present) from contralateral region	FNA4
FNA, Normal/hyperplastic prostate from a contralateral region	FNA5
FNA, Sextant FNA and core biopsies from the peripheral zone of the right and left apex, midgland and base	FNA6
Core biopsy cells, Tumor	CC1
Core biopsy cells, Area adjacent to the tumor	CC2
Core biopsy cells, Normal/hyperplastic prostate from an ipsilateral periurethreal region	CC3
Core biopsy cells, Tumor (if present) from contralateral region	CC4
Core biopsy cells, Normal/hyperplastic prostate from a contralateral region	CC5
Core biopsy cells, Sextant FNA and core biopsies from the peripheral zone of the right and left apex, midgland and base	CC6



2. Prostate Core biopsy cells storage

Prostate Core biopsy cells storage box map card

1	2	3	→					
10	→							
19								

3. Prostate FNA cells storage

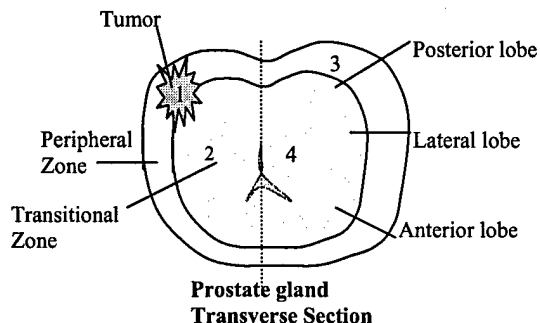
Prostate FNA cells storage box map card

1	2	3	→					
10	→							
19								



Procedure for collecting tissue for tissue section

1. Tissue must be sliced and placed in labeled appropriate vials within 30 minutes of resection.



2. Identify the prostate gland orientation and the cancer location.
 - 1) Tumor
 - 2) Area adjacent to the tumor
 - 3) Area not adjacent to the tumor from an ipsilateral peripheral zone
 - 4) Normal/hyperplastic prostate from an ipsilateral periurethral region
 - 5) Tumor (if present) from contralateral region, otherwise area from an contralateral peripheral zone
 - 6) Area adjacent to the tumor (if present) from contralateral peripheral region, otherwise area from another contralateral peripheral zone
 - 7) Normal/hyperplastic prostate from a contralateral region
3. Slice each piece from different site; each piece should weigh 200-30 mg.
Leave out prostate capsule.
4. Pieces of tissue (including #3, 6) will be placed in a pre-labeled cryogenic vial following by flash frozen in liquid nitrogen.
5. Pieces (including #1, 2, 4, 5, 7) will be place in a pre-labeled 2.0 ml vials containing formalin.



6. All the tissue will be transported to the QFIA lab, snap frozen tissue will be stored in -80°C freezer followed by appropriate labeling. Formalin fixed tissue will be stored in room temperature with appropriate labeling.
7. Enter storage information into **TISSUE INVENTORY** database.

Tissue Vial Labeling and Storage

1. Each vial of tissue is labeled with
 - case number
 - tissue code
 - storage date

<u>Tissue Description</u>	<u>Tissue Code</u>
Tumor	FT1
Area adjacent to the tumor	FT2
Area not adjacent to the tumor from an ipsilateral peripheral zone	FT3
Normal/hyperplastic prostate from an ipsilateral periurethreal region	FT4
Tumor (if present) from contralateral region, otherwise area from an contralateral peripheral zone	FT5
Area adjacent to the tumor (if present) from contralateral peripheral region, otherwise area from another contralateral peripheral zone	FT6
Normal/hyperplastic prostate from a contralateral region	FT7

2. Frozen Tissue Storage
The cardboard storage box contains a 9X9 grid and can be filled as indicated in the following map card. Verify that each tube is labeled correctly. The inventory list sheet should be completed each time.



Prostate frozen tissue storage box map card

1	2	3	→					
10	→							
19								

3. Formalin Fixed Tissue storage

The 2.0 ml vials containing formalin and tissue samples will be stored at room temperature in a cryo storage box. The caps of these vials must be secured and the label of each vial should be verified. The storage box contains a 9X9 grid and can be filled as indicated in the following map card. The inventory list sheet should be completed each time. These vials should not be exposed to extreme changes in temperature and are kept at room temperature until they are processed.

Prostate formalin fixed tissue storage box map card

1	2	3	→					
10	→							
19								



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Notes and Comments



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PC-3 PROSTATE CANCER CELL LINE: PASSAGING AND CRYOPRESERVATION												
CELL PASSAGING												
Operator Name	Passage (Psg)		Cell		Culture Medium			Culture Vessels				
	Date	Number	Harvest	Source Cat# Lot#	Basal	Serum	Batch	Type	Size	# cells/flask	Number	
George P. Casale	6/4/2003	6	NA	passage 6 cryopreserved 3/10/03	F12K	5% FBS	FK-003	T-Flask	25 cm ²	7.5 M	1	
George P. Casale	6/5/2003	7	6 M/flsk	Psge 6	F12K	5% FBS	FK-003	T-Flask	75 cm ²	1.2 M	2	
George P. Casale	6/9/2003	8	4.5M/flsk	Psge 7	F12K	5% FBS	FK-003	T-Flask	75 cm ²	1.4 M	4	
George P. Casale	6/13/2003	9	7.3M/flsk	Psge 8	RPMI1640	5% FBS	RP-001	T-Flask	75 cm ²	1.4 M	6	
George P. Casale	6/17/2003	10	7.1M/flsk	Psge 9	RPMI1640	5% FBS	RP-001	T-Flask	75 cm ²	1.4 M	1	
George P. Casale	6/21/2003	11	7.3M/flsk	Psge 10	RPMI1640	5% FBS	RP-001	T-Flask	75 cm ²	1.4 M	4	
George P. Casale	6/26/2003	12	5.5M/flsk	Psge 11	RPMI1640	5% FBS	RP-002	T-Flask	75 cm ²	1.4 M	1	
George P. Casale	7/25/2003	10	NA	passage 9 cryopreserved 6/17/03	RPMI1640	5% FBS	RP-002	T-Flask	75 cm ²	NA	1	
George P. Casale	7/28/2003	11	11M/flsk	Psge 10	RPMI1640	5% FBS	RP-002	T-Flask	75 cm ²	2.0 M	5	
George P. Casale	7/31/2003	12	5.5M/flsk	Psge 11	RPMI1640	5% FBS	RP-002	T-Flask	75 cm ²	1.4 M	2	
George P. Casale	8/4/2003	13	ND	Psge 12	RPMI1640	5% FBS	RP-002	T-Flask	75 cm ²	ND	6	
George P. Casale	8/7/2003	14	ND	Psge 13	RPMI1640	5% FBS	RP-002	T-Flask	75 cm ²	ND	1	
<div>Labeling: *PC-3 HL *Seed date *Number of cells seeded where M=> million *Passage number</div>												
<div>Comments: Seeded 1.4 M / 75cm²: 3 days to near confluence. Coulter set at >10 microns = cell count. Passage on 6/26/03: cell numbers were clearly low in one flask. Terminate passage 12! NA=> Not Applicable ND=> Not Determined NR=> Not Recorded</div>												

PC-3 PROSTATE CANCER CELL LINE: PASSAGING AND CRYOPRESERVATION												
CELL PASSAGING												
Operator Name	Passage (Psge)		Cell		Culture Medium			Culture Vessels				Number
	Date	Number	Harvest	Source Cat# Lot#	Basal	Serum	Batch	Type	Size	# cells/flask		
Dali Huang	8/13/2003	15	ND	Psge 14	RPMI1640	5% FBS	RP-003	T-Flask	75 cm ²	1:4 for 1; 1:1 split for 2		3
Dali Huang	8/17/2003	16	5M/flask	Psge 15	RPMI1640	5% FBS	RP-003	T-Flask	75 cm ²	1.4 M		1
Dali Huang	8/21/2003	17	6.4M/flask	Psge 16	RPMI1640	5% FBS	RP-004	T-Flask	75 cm ²	1.4 M		1
Dali Huang	8/25/2003	18	8.3M/flask	Psge 17	RPMI1640	5% FBS	RP-004	T-Flask	75 cm ²	1.4 M		1
Dali Huang	8/29/2003	19	6.89M/flask	Psge 18	RPMI1640	5% FBS	RP-004	T-Flask	75 cm ²	1.3 M		1
Dali Huang	9/2/2003	20	8.0M/flask	Psge 19	RPMI1640	5% FBS	RP-004	T-Flask	75 cm ²	1.4 M		1
Dali Huang	9/5/2003	21	5.0M/flask	Psge 20	RPMI1640	5% FBS	RP-004	T-Flask	75 cm ²	1.4 M		1
Lance Wiebusch	9/8/2003	22	5.49M/flask	Psge 21	RPMI1640	5% FBS	RP-004	T-Flask	75 cm ²	1.2 M		1
Lance Wiebusch	9/12/2003	23	8.1M/flask	Psge 22	RPMI1640	5% FBS	RP-005	T-Flask	75 cm ²	1.4 M		1
Dali Huang	9/15/2003	24	6.1M/flask	Psge 23	RPMI1640	5% FBS	RP-005	T-Flask	75 cm ²	1.4 M		1
Lance Wiebusch	9/18/2003	25	6.83M/flask	Psge 24	RPMI1640	5% FBS	RP-005	T-Flask	75 cm ²	1.4M for 1 1.0 M for 2		2
Dali Huang	9/22/2003	26	6.78M/flask	Psge 25	RPMI1640	5% FBS	RP-005	T-Flask	75 cm ²	1.4 M		1
Comments:								Labeling:				
Seeded 1.4 M / 75 cm ² T-flask: 3 days to near confluence. Coulter set at >10 microns = cell count.								*PC-3 HL				
NA=> Not Applicable								*Seed date				
ND=> Not Determined								*Number of cells seeded where M=> million				
NR=> Not Recorded								*Passage number				

PC-3 PROSTATE CANCER CELL LINE: PASSAGING AND CRYOPRESERVATION										
CRYOPRESERVATION										
Operator Name	Cell Passage		Medium			Cryo Vials				
	Date	Number	Identity	Vendor	cat. # Lot #	Rec'd Opened Expir Date	In Storage Removed	Identification	Location	
George P. Casale	2/28/2003	3	Origen DMSO Freeze Medium	Fisher Scientific	210002 37475	01-14-03 02-23-03 08-01-05	3 / 0	PC-3;P4(3) 02-28-03	Locator 8a Rack 1;Box 1; slots 8-10	
George P. Casale	3/14/2003	6	Origen DMSO Freeze Medium	Fisher Scientific	210002 37475	01-14-03 02-23-03 08-01-05	2 / 1	PC-3;P7(6) 03-14-03	Locator 8a Rack 1;Box 1; slots 11-12	
George P. Casale	6/17/2003	9	Origen DMSO Freeze Medium	Fisher Scientific	210002 37475	01-14-03 02-23-03 08-01-05	5 / 1	PC-3;P9 06-17-03	Locator 8a Rack 1;Box 1; slots 13-16	
George P. Casale	6/26/2003	11	Origen DMSO Freeze Medium	Fisher Scientific	210002 37475	01-14-03 02-23-03 08-01-05	3 / 0	PC-3;P11 06-26-03	Discarded these vials; cf note below	
Regarding preservation on 6/26/03, see note for cell passage on 6/26/03. Discard vials prepared on 6/26/03!										

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Abstract accepted to the AACR, January 25-29, 2003, Waikoloa, Hawaii

Authors: Dali Huang , George P. Casale, Nizar K. Wehbi, George P. Hemstreet III

Tissue Transglutaminase Down-regulation: a Potential Biomarker for Prostate Cancer Premalignancy

Previous studies indicate that there are differences in the expression of tissue transglutaminase (tTGase) in prostate cancer tissues when compared to benign prostatic hyperplasia (BPH) utilizing image analysis and conventional immunohistochemical staining.^{1,2} In these studies, there was also a visual down regulation of tTGase in PIN lesions and a suspected down regulation of tTGase in the normal appearing glands of cancer-bearing prostates. Based on the concept of biochemical field disease, we previously demonstrated that field changes can predict the development of bladder cancer 3-5 years prior to clinically manifest disease, utilizing quantitative fluorescence image analysis (QFIA).^{3,4} In order to further discriminate subtle down regulation of this biomarker in the normal appearing cells in the premalignant field and as a first step towards developing a male PAP test, we quantified the expression of tTGase in archival paraffin-embedded tissues from 6 cases of prostate cancer and 6 cases of BPH.

Materials and Methods: Age-matched archived tissue blocks (6 cases of BPH and 6 cases of prostate cancer) were obtained from the department of Pathology/Microbiology at the University of Nebraska Medical Center. Tissue sections were deparaffinized, re-hydrated, processed for antigen retrieval, and stoichiometrically labeled for tissue transglutaminase with a specific monoclonal antibody in conjunction with a biotinylated secondary antibody and streptavidin-AlexaFluor[®]488. Fluorescence images were captured and analyzed by a Leica automated microscope system and Image-Pro Plus software. Differences between group means were statistically evaluated by Student's t-Test.

Results: The frequency of labeled glands was higher in the BPH specimens (41%) compared to the cancer specimens (17%; $P < 0.05$). Within cancer-bearing glands, labeling frequency was significantly higher ($P < 0.05$) in the normal-appearing areas (46%) compared to cancerous areas (7%). There was no difference between BPH specimens and normal-appearing areas of the cancer specimens. Mean labeling intensity (540) of positive glands in the BPH specimens was elevated in comparison with labeling (420) of positive glands in the cancer cases, but did not achieve statistical significance ($P = 0.21$). There was also no statistical difference between normal-appearing and cancerous areas of cancer-bearing glands. The mean intensity of all positive BPH glands (342 from 6 cases) was greater than that of all positive glands in either the normal-appearing (625 glands) or cancerous areas (236 glands) of the 6 cancer cases ($P < 0.001$). There was no difference, however, between the normal-appearing and cancerous areas of cancer-bearing glands.

Conclusions: These preliminary results with age-matched prostate cancer and BPH cases support the concept of field effect or field disease in normal-appearing glandular tissue in cancer-bearing prostates, and the application of QFIA to single-cell proteomics to

establish a biomarker profile that identifies patients with an initially negative prostate biopsy, who require rebiopsy. This biomarker profile could be used to determine the risk of developing biologically active prostate cancer, detect early disease, and monitor effectiveness of chemopreventive agents. 1) Cancer, **89**: 412, 2000. 2) J Urol, **167**: 2215, 2002. 3) J Natl Cancer Inst, **93**: 427, 2001. 4) Proc Natl Acad Sci U S A, **90**: 8287, 1993

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Abstract submitted to the AACR, March 27-31, 2003, Orlando, Florida

**A MONOCLONAL ANTIBODY FOR MACROMOLECULAR ADDUCTS OF
ESTRADIOL-3,4-QUINONE, A SUSPECTED INITIATOR OF BREAST AND
PROSTATE CANCERS**

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Epidemiology and laboratory animal studies have established a linkage between breast and prostate cancers and estrogen exposure. Mammalian tissues metabolize 17-beta-estradiol (E_2) to the catechols 2-hydroxy E_2 (2-OHE $_2$) and 4-hydroxy E_2 (4-OHE $_2$). Recent studies support a close association between preferential 4-hydroxylation of E_2 and estrogen dependent carcinogenesis (E_2 -DC). In model systems, organs susceptible to E_2 -DC express estrogen 4-hydroxylase (CYP1B1) and produce more 4-OHE $_2$ than 2-OHE $_2$. Organs resistant to E_2 -DC do not express CYP1B1 and produce predominantly 2-OHE $_2$. Catechol estrogens can be oxidized to quinones that bind covalently to DNA and proteins. The present work is based on the hypothesis that tissues engaged in E_2 -DC, produce E_2 -3,4-quinone (E_2 -3,4-Q) in amounts sufficient to escape detoxification and form DNA and protein adducts that participate in carcinogenesis. Further, E_2 -3,4-Q adducted to cellular proteins may serve as a biological marker of "escaped" E_2 -3,4-Q and risk of both breast and prostate cancers.

We have developed a monoclonal antibody (Mab) with high specificity and affinity ($K_a = 0.5 \times 10^8 \text{ M}^{-1}$) for E_2 -3,4-Q adducted to any cellular macromolecule.

Immunohistochemical staining with the antibody produced intense, specific labeling in slide preparations of rat breast tissue treated with E_2 -3,4-Q, no detectable labeling of tissues treated with E_2 -2,3-Q and little or no background. In addition, the antibody produced a specific fluorescence signal in PC3 prostate cancer (PC) cells exposed *in vitro* to 4-OHE $_2$ (nominal 10 micromolar), but no signal in similarly treated DU-145 PC cells. In conclusion, this newly developed antibody may serve as a unique and sensitive probe to assess 1) a plausible mechanism of breast and prostate carcinogenesis, and 2) the E_2 -3,4-Q-protein adduct as a biomarker for both breast and prostate cancer risk. Funded by the U.S. Department of Defense.

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PHENOTYPIC BIOMARKER PROFILES FOR INDIVIDUAL RISK ASSESSMENT

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Introduction and Objective: We have previously demonstrated that prostate and bladder cancer express premalignant molecular fingerprints in an organ at risk for cancer.^{1,2} The objective of this presentation is to provide a succinct overview of our experience with the selection of biomarkers for prostate cancer and the relevance of estrogen related oxidative stress in prostate carcinogenesis. The present work is based on the concept of molecular field disease and the hypothesis that prostate glands, engaged in E₂-DC, produce E₂-3,4-quinone (E₂-3,4-Q) in amounts sufficient to escape detoxification and form DNA adducts essential for the development of prostate cancer. **Methods:** Quantitative Fluorescence Image Analysis (QFIA) was used to measure three high-level phenotypic biomarkers: pan-cadherin, tTgase, and G-actin.³⁻⁵ Prostate tissues from 38 patients with adenocarcinoma of the prostate and 33 controls with 10-year follow up were stained for tTgase and pan-cadherin. A specific monoclonal antibody (Mab) for single-cell analysis of E₂-3,4-Q has been developed to detect oxidative changes in DU145 cells and prostate tissues. Biomarkers were measured in prostate tissues with conventional IHC and with fluorescence nanocrystal technology. **Results:** 97.4% of the cases (37/38) were detected using a profile of both biomarkers. tTgase has also been demonstrated to be down regulated in the premalignant field reflecting biochemical field disease. The affinity constant ($0.5 \times 10^8 \text{ M}^{-1}$) for binding to E₂-3,4-Q was determined by competitive ELISA. Immunohistochemical (IHC) staining with the antibody produced intense and specific staining of tissue sections treated with E₂-3,4-Q with no detectable cross-reactivity to E₂-2,3-Q and little or no background. **Conclusions:** Using a panel of high-level biomarkers provides a higher sensitivity and specificity and could be used to screen individuals at risk for bladder and prostate cancer. Single-cell proteomics facilitates the study of genetic instability and epigenetic signaling (stromal-epithelial interactions) in relation to cancer therapy and diagnosis. In conclusion, this newly developed QFIA techniques allows the detection of molecular fingerprints in cells but also permits a unique and sensitive evaluation of 1) a plausible mechanism for initiation of non-familial prostate cancer and 2) the E₂-3,4-Q-protein adduct as a biomarker of prostate cancer risk. 1) Proc Natl Acad Sci U S A, **90**: 8287, 1993. 2) J Natl Cancer Inst, **93**: 427, 2001. 3) Cancer, **89**: 412, 2000. 4) Cancer Detect Prev, **24**: 464, 2000. 5) J Urol, **167**: 2215, 2002